ENTRAPPING OF HYDROPHOBIZED PLAGUE CAPSULAR ANTIGEN INTO THE LARGE UNILAMELLAR LIPOSOMES

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Liposomes, having adjuvant properties increase the body's /90** immune response to the antigens introduced with them [1]. The immunopotentiating effect depends on the quantity of antigen immobilized in the liposomes, the presence of it on the outer surface of the membrane, lipid composition, size of the vesicles and other conditions [2]. Hydrophilic proteins are adsorbed on the liposome membrane in a small quantity, which reduces immunogenicity of the preparations. Consequently the problem emerged of immobilizing proteins on the outer surface of the vesicles.

Diverse methods have been developed in recent years for fixing proteins on the liposome membrane [3]. In the majority of cases different methods of covalent bonding of proteins with phospholipids are used, which is not at all desirable for immobilization of antigens insofar as it could cause production of antibodies on the phospholipid-protein complex.

Torchilin, et al. [4, 5] achieved a significant increase in protein inclusion in the lipid membrane of "cholate" liposomes through preliminary hydrophobization of α-chemotrypsin by palmitic acid chloroanhydride. Shen De Phen, et al. [6] further suggested a version of this method which makes it possible to build monoclonal antibodies modified by N-oxysuccinimide ether of palmitic acid into the liposomes prepared by the method of "phase inversion." We studied the effectiveness of including palmitoylized capsule antigen of

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the plague microbe into liposomes prepared by detergent dialysis and "phase inversion", as well as the impact of the lipid composition of membranes on fixing of the hydrophobized antigen.

Materials and Method

Capsule antigen (fraction I) was isolated by the method of Baker, et al. [7] from acetone-dried bacterial mass of Y. pestis EV-79 grown on an agarized Hottinger medium with pH 7.2 at 37°C for 48 h. Serological activity of the antigen was assessed in the reaction of direct hemagglutination with antigel plague erythrocytic diagnosticum and in the reaction of antibody neutralization with the antigen erythrocytic diagnosticum and plague agglutinating serum. Fraction I was marked by iodine ($Na^{125}I$ of the association Izotop) by the method of Greenwood and Hunter [8]. Preparation radioactivity was measured using the RIA Gamma gamma counter (LKB-Wallack, Sweden). The protein was hydrophobized by palmitoyl chloride (Fluka, Switzerland) by the original method of Torchilin, et al. [4] with a modification consisting of reduction in the concentration of sodium desoxycholate in the reaction medium to 0.15% [6]. In a number of experiments the premarked [^{125}I] antigen was palmitoylized. Palmitoylizing of the antigen essentially does not influence its serological activity, as confirmed by the results of the reaction indirect hemagglutination with free and palmitoylized antigen.

In order to prepare the liposomes, the "lecithin-standard" produced by the Kharkov plant of bacterial preparations, cholesterol and dicethylphosphate (Serva, FRG), phosphatidilserin and Sphyngomyelin obtained from pig brain were used. The organic solvents used were freshly distilled, dehydrated

acetone, butanol, methanol and chloroform. The liposomes were prepared by methods of detergent dialysis [4] and "phase inversion" [9]. Small oligolaminar liposomes were obtained from 50 μmole of a mixture of egg lecithin, cholesterol and dicephylphosphate in a molar ratio of 5:4:1 in 1 ml of 0.01 M of phosphate buffer, with pH 7.2 containing 1% sodium desoxycholate and 0.1% antigen. Fraction I was used both in the native and in the modified state.

Large oligolaminar liposomes were obtained by the method of Szoka, et al. [9] using 0.01 M phosphate buffer containing 0.15% sodium desoxycholate and 0.25% antigen solution. The nonincluded antigen was separated using centrifuging (100,000 g; 1 h), the liposomal sediment was washed with 0.01 M of phosphate buffer with pH 7.2.

In some cases gel-chromatography was used on sepharose CL-4B (Pharmacia, Sweden). A total of 1 ml of liposome suspension was added to a column of size 1.6 x 30 cm eluated with buffer at a rate of 0.3 ml/min and a fraction of volume 2.0 ml was collected.

In order to assess the adsorption of antigen on the membrane surface of the liposomes 0.25 ml of liposome suspension consisting of egg lecithin, cholesterol and dicephylphosphate in a 7:2:1 correlation with concentration of lipids 60 μmole/ml, was incubated for 48 h at a temperature of 37°C with 0.25 ml of a 0.25% solution of marked antigen. At the end of incubation, liposomes were separated from the free protein, using gel-chromatography on minicolumns filled with CL-4B sepharose. The quantity of adsorbed antigen was determined radiometrically. Protein content in the liposomes was assessed by the quantity of radioactive marker ^{125}I. The

quantity of antigen fixed on the outer surface of the liposomal membrane was determined by its specific activity in reactions of indirect hemagglutination or antibody neutralization, total serological activity was assessed after destruction of the /91
vesicles by an acetone-butanol mixture 3:1 which was added in an equal volume to the liposomal suspension. The minimum quantity of antigen detected in the reaction of indirect hemagglutination was 33×10^{-9} g.

Results and Discussion

We previously demonstrated [10] the advantage of the method of detergent analysis to prepare liposomes containing free capsule antigen compared to other methods of obtaining small monolamellar vesicles.

In the preliminary experiments we compared the effectiveness of including and building into the liposomal membrane prepared by detergent dialysis of free and palmitoylized fraction I. Inclusion of the palmitoylized fraction I into the liposomes was reliably higher than free (Table 1). According to the data of serological studies, the quantity of antigen included in the internal volume of the vesicles in both cases was approximately the same. The main difference was the content of palmitoylized protein on the outer surface of the liposomes where there is 3.7 times more than free. The findings confirm the advantage of using hydrophobized protein to fix it on the surface of the lipid membrane.

The effectiveness of including antigen in the liposomes could be significantly increased when the method of "phase conversion" is used. The use of this procedure to encapsule the palmitoylized protein prepared by the original technique

[4] in a 1% solution of sodium desoxycholate, however, is impossible because of the high content of detergent. Consequently in order to obtain liposomes we used a solution of palmitoylized fraction I in the presence of 0.15% solution of desoxycholate as described for N-oxysuccinimide ether of palmitic acid [6]. The technique of palmitoylizing does not differ from that described by Torchilin, et al. [4] with the exception of the fact that the solution of palmitoylized protein is dialyzed versus 0.15% sodium desoxycholate that does not cause liposomal destruction. At the same time, decrease in detergent concentration does not lead to a significant decrease in solubility of hydrophobized protein.

TABLE 1. INCLUSION OF FREE AND PALMITOYLIZED CAPSULE ANTIGEN OF PLAGUE MICROBE IN LIPOSOMES (M±m; n = 3 - 5) (Key on next page)

(1) Липидный состав липосом		(2) Характер антигена	Содержание антигена (в мкг/100 мкмоль липидов) (3)		
			(4) В препарате	(5) Во внутреннем объеме	(6) Снаружи на мембране
		(7) «Холатные» липосомы			
1. (12) Яичный лецитин : холестерин : дицетилфосфат = 5 : 4 : 1		(9) гидрофобизованный	220,4±35,6	73,0±14,2	147,4±23,6
2. То же (11)		(10) свободный	112,3±22,3*	72,1±11,1	40,3±12,9*
		(8) Большие олигослойные липосомы			
3. » »	7 : 2 : 1	(10) свободный	526,5±41,7**	447,0±38,9**	79,5±24,9
4. » »	7 : 2 : 1	гидрофобизованный (9)	698,6±82,2*	441,6±41,2*	257,6±39,8
5. » » (13)	5 : 4 : 1	То же (11)	592,4±85,6*	198,5±31.1**	393,9±56,7*
6. Фосфатидилсерин : сфингомиелин : холестерин = 1 : 4 : 5		» »	527,3±65,9*	210,6±28,1*	316,7±40,3*

TABLE 1. Key:
1. lipid composition of liposomes
2. nature of antigen
3. content of antigen (μg/100 mole of lipids)
4. in preparation
5. in internal volume
6. from the outside on the membrane
7. "cholate" liposomes
8. large oligolaminar liposomes
9. hydrophobized
10. free
11. the same
12. egg lecithin:cholesterol:dicephylphosphate
13. phosphatidylserin:sphyngomyelin:cholesterol
1* **Differences compared to preparations No 1 and previous preparation are reliable ($p < 0.05$).

The percentage inclusion into liposomes of palmitoylized /92 antigen is reliably greater than free, and is respectively 46.1 ± 5.4 and 34.7 ± 2.7%. The content of fraction I inside and on the outer surface of the vesicles depends on the physical and chemical properties of the protein and the lipid composition of the liposomes (see Table). Large oligolaminar liposomes prepared from egg lecithin, cholesterol and dicephylphosphate in a molar ratio of 7:2:1, with the same effectiveness captured in the internal volume free and palmitoylized fraction I. At the same time the hydrophobized antigen has a great affinity for the lipid membrane, as a result of which its quantity on the outer surface of the liposome is 3.2 times greater than the free.

Higher content of cholesterol and replacement of egg lecithin by sphyngomyelin led to the formation of "hard" liposomes characterized by lower inclusion of protein. In this case the quantity of hydrophobized antigen fixed on the outer surface of the liposomes is somewhat higher than for vesicles from egg lecithin with small content of cholesterol. This is possibly related to the larger area of the total

surface of "solid" liposomes because of decrease in the size of vesicles and increase in their quantity. On the other hand, low mobility of the lipid molecules in the membrane of "hard" liposomes could limit desorption of the antigen on the outer surface.

In order to assess the affinity of palmitoylized free fraction I to the liposome membrane, we conducted a series of experiments to study the capacity for adsorption of protein on the surface of "empty" liposomes and competition of the hydrophobized and free molecules of the antigen for bonding with the lipid membrane. In studying exchange of protein molecules on the surface of a lipid membrane, we used liposomes containing antigen unmarked by radioactive iodine (Table 2). Adsorption of the palmitoylized fraction I on the surface of the "empty" liposomes is 4.3 times greater than adsorption of the free antigen. The high affinity of the hydrophobized protein for the lipid membrane was revealed during incubation of the marked palmitoylized fraction I with liposomes containing free antigen. In this case on the liposomal surface there is significant adsorption of the palmitoylized antigen, despite the fact that the vesicles already contain protein. When the liposomes are loaded by palmitoylized fraction I, their incubation with marked free antigen leads to a slight inclusion of it into the lipid membrane. The mechanism for fixing antigen on the membrane during preparation and during incubation with the finished liposomes apparently differs. The quantity of free and hydrophobized antigen adsorbed on the "empty" liposomes is respectively 15.1 and 20.3% of the antigen content on the surface of the liposomes prepared with protein. It is possible that because of difference in the methods of fixing the antigen on the liposomal surface containing fraction I

in free or palmitoylized form, additionally 10.4 and 15% antigen are adsorbed in the corresponding form. In addition to exchange and competition of the antigen contained in the liposomes and in the solution, independent bonding mechanisms /93 are also possible.

TABLE 2. ADSORPTION OF ANTIGEN MARKED BY ^{125}I ON LIPID MEMBRANE DURING INCUBATION WITH ANTIGEN-CONTAINING LIPOSOMES (μg of antigen per 100 μmole of lipid)

Composition of Incubation Mixture	Quantity of Adsorbed Marked Antigen
"Empty"liposomes and fraction I marked by ^{125}I	12.0
The same and palmitoylized fraction I marked by ^{125}I	52.2
Liposomes containing fraction I and fraction marked by ^{125}I	8.3
The same and palmitoylized fraction I marked by ^{125}I	43.3
Liposomes containing palmitoylized fraction I and fraction I marked by ^{125}I	2.2
The same and palmitoylized fraction I marked by ^{125}I	38.4

The high affinity of palmitoylized protein for the membrane has been confirmed in experiments to study the exchangeability of hydrophobized antigen molecules contained on the surface of the liposomes and added to the incubation mixture. This process was more pronounced than the case of the use of free antigen.

These studies graphically demonstrated the significant affinity of palmitoylized capsule antigen of plague microbe for the lipid membrane and the high competitiveness for the bonding site with liposomes compared to the free antigen.

Hydrophobization of capsule antigen of the plague microbe by palmitoyl chloride thus made it possible to greatly improve its affinity for the lipid membrane and to more effectively include protein into the liposomes, mainly on their outer surface maintaining its serological activity.

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